Validation of a new genotoxicity pre-screening package for food additives

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Background and purpose

Increased food demand due to a growing global population incentivises the need to identify and develop safer and more effective food additives as required by regulators such as the FDA and EFSA[1]. *In vitro* genotoxicity studies are required by these organisations of which assays, such as the Comet assay, present lack of consistency in the literature regarding incubation time and analysis. The possible identification of DNA-damage-inducing food additives using automated high content screening with robust data analysis would support de-risking food additives.

In general, genotoxins are chemicals that cause DNA or chromosomal damage. They can be assessed using *in vitro* assays like phosphorylation of histone H2AX (pH2AX) and histone H3 (pH3), and *in vitro* micronucleus (MNT) as described in OECD guideline number 487. As pH2AX is a marker for direct DNA damage caused by clastogens, with aneugens having been shown to modulate levels of pH3, this method allows to identify genotoxins with clastogenic and aneugenic classification[2,3]. MNT are formed from the misincorporation of chromosomal material that might be structurally and/or genetically damaged, due to interactions with clastogens and/or aneugens.

Methods

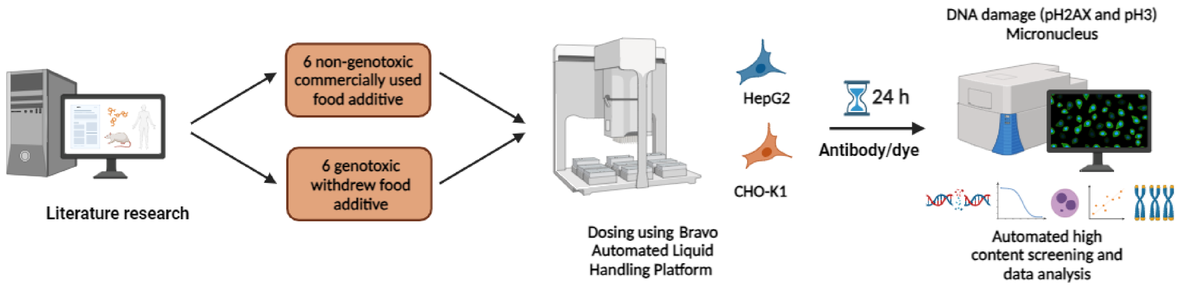

Figure 1: Workflow of the genotoxicity pre-screening package validation. 6 non-genotoxic commercially used food additives and 6 genotoxic withdrew food additives were selected based on literature research to validate these assays. 8 [DNA damage (pH2AX and pH3)] or 10 concentrations (MNT) were selected for each compound and a concentration curve established, ranging from 10x the top concentration found in literature to 2500x lower. Cells were seeded in 384-well plate for 24h and dosed using Bravo automated Liquid handling platform for another 24h. Immunohistochemistry was then performed, and cells imaged using CellInsight CX7 (ThermoFisher) and analysed using automated algorithms set up in HCS Studio software (ThermoFisher). Workflow created using Biorender.

DNA damage (pH2AX and pH3) assay

- HepG2 cells were seeded and let to adhere for 24h and then dosed with 12 reference food additives for another 24h.
- Cells were fixed and stained against H2AX phosphorylated Ser139 and H3 phosphorylated Ser10 to identify clastogen and aneugen features. Hoechst 33342 was used to assess cell count, nuclear size, and DNA structure for cytotoxicity assessment.

In vitro micronucleus (based on OECD 487)

- CHO-K1 cells were seeded and let to adhere for 24h and then dosed with 12 reference food additives for another 24h.
- Next, cells were incubated with cytochalasin B (actin polymerization inhibitor) prior to mitosis to originate binucleated cells, allowing the identification of micronuclei only in cells that completed one mitosis.
- Cells were stained with Hoechst 33342 to assess cell number and binucleated cells. Cell health was assessed by using a permeability dye. Cytokinesis block proliferation index and cytostasis were also calculated based on cell count and number of binucleated cells.

References

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2. Khoury L et al. (2016) *Arch Toxicol* 90(8):1983–95.
3. Kuo et al. (2008) *In vivo* 22(3):305-9.
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5. Pandir (2016) Cytotechnology 68:725-733
6. Soares et al. (2015) *Anticancer Research* 35 (3) 1465-1474
7. Bair (2001) *Int J Toxicol* 20(3)23-50
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Results

The results of this study predicted the genotoxicity of already characterised food additives by 75% (pH2AX and pH3) and 67% (MNT). Potassium bromate was the only genotoxic control with a response in both assays, being classified as clastogen (pH2AX fold change (FC) > 1.5) (**Figure 2**) and induced MNT formation (**Figure 3**). Other controls were classified as genotoxic in just one of the assays: chloroacetic acid that was classified as an aneugen (pH2AX and pH3 FC > 1.5) (**Figure 2**) in the pH2AX and pH3 assay, and propylparaben that induced MNT formation (**Figure 4**).

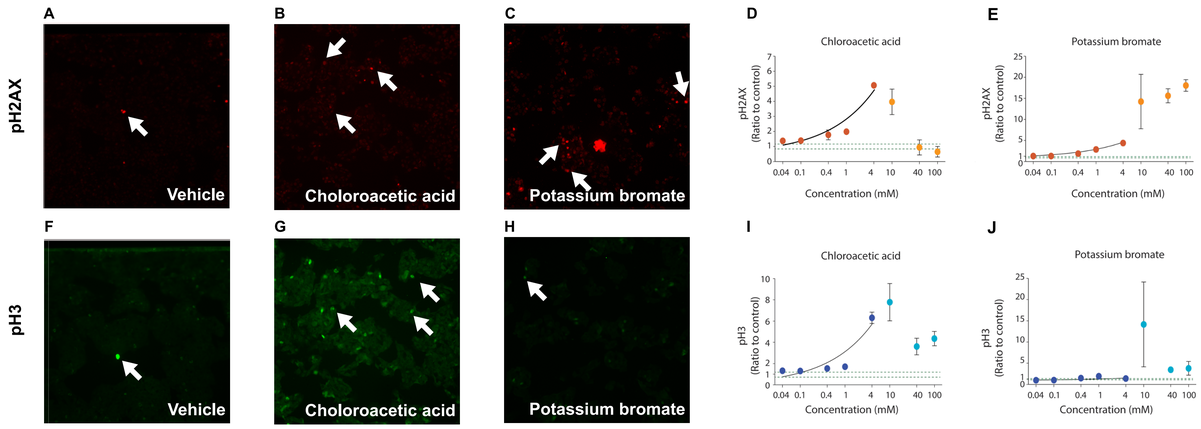

Figure 2: Chloroacetic acid and potassium bromate induce alterations in pH2AX and pH3 in HepG2 cells. Representative images of HepG2 cells stained with anti-pH2AX (A to C) or anti-pH3 (F to H) antibodies after 24h incubation with reference food additives, and representative graphs of respective quantification for chloroacetic acid (D or I) and potassium bromate (E or J). Each dot is an average of 3 technical replicates. Black line is the exponential regression fitting curve. Light colours correspond to data that was not included in the fitting due to > 50% cell loss (data now shown). Green dashed lines represent vehicle data distribution. All experiments were run independently at least three occasions. Arrows are indicating the foci used for quantification.

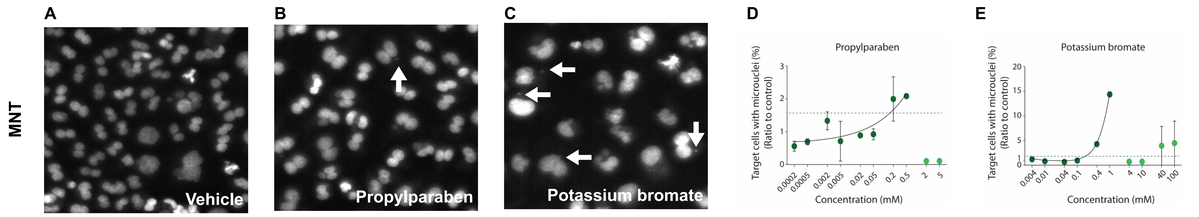

Figure 3: Propylparaben and potassium bromate induce micronuclei in CHO-K1 cells. Representative images of CHO-K1 cells stained with Hoechst 33342 (A to C) after 24h incubation with reference food additives and 27.5h with Cytochalasin B, and representative graphs of respective quantification for propylparaben (D) and potassium bromate (E). Each dot is an average of 3 technical replicates. Black line is the exponential regression fitting curve. Light colours correspond to data that was not included in the fitting due to > 50% cell loss (data now shown). Green dashed lines represent vehicle data distribution. All experiments were run independently at least three occasions. Arrows are indicating the presence micronucleus.

	Genotoxic in literature?	DNA damage	MNT positive	Positive in one assay
Ascorbic acid	NO	NO	NO	NO
MSG	NO	NO	NO	NO
Sodium benzoate	NO	NO	NO	NO
Potassium sorbate	NO	NO	NO	NO
Aspartame	NO	NO	NO	NO
Quinoline Yellow	YES - *in vitro* NO - *in vivo*	YES	NO	YES
Cinnamyl anthranilate	YES	YES	NO	YES
Propylparaben	YES	NO	YES	YES
Benzoic acid	YES	NO	NO	NO
Chloroacetic acid	YES	YES	NO	YES
Potassium bromate	YES	YES	YES	YES
Tartrazine	YES	NO	NO	NO
Matches with literature:		**75%**	**67%**	**83%**

Table 1: Data summary for 12 reference food additives assessed by both genotoxicity assays.

Conclusion

- This approach includes two methods to identify genotoxicity in food additives, using automated and high-throughput approaches. From the 12 reference food additives analysed, we predicted correctly the genotoxicity of 10 (83%) in at least one of the methods. Both false negatives (benzoic acid and tartrazine) have been shown to induce DNA damage under certain conditions[5, 6], but not in others[7, 8]. As some of the methods and the dosing in the literature differ from ours, and the disparity in the literature, this could explain the data for these two compounds.
- As our assays are standardised, automated and use robust algorithms for data analysis, they bypass issues other methods might present. In addition, these methods also allow the detection of epigenetic changes and identification of aneugens and clastogens. Besides the genotoxicity markers, these assays also provide information about cell survival, membrane integrity and cell cycle information.